Saturated and Polyunsaturated Fatty Acids Reciprocally Modulate Dendritic Cell Functions Mediated through TLR4[1]

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TLRs provide critical signals to induce innate immune responses in APCs such as dendritic cells (DCs) that in turn link to adaptive immune responses. Results from our previous studies demonstrated that saturated fatty acids activate TLRs, whereas *n*-3 polyunsaturated fatty acids inhibit agonist-induced TLR activation. These results raise a significant question as to whether fatty acids differentially modulate immune responses mediated through TLR activation. The results presented in this study demonstrate that the saturated fatty acid, lauric acid, up-regulates the expression of costimulatory molecules (CD40, CD80, and CD86), MHC class II, and cytokines (IL-12p70 and IL-6) in bone marrow-derived DCs. The dominant negative mutant of TLR4 or its downstream signaling components inhibits lauric acid-induced expression of a CD86 promoter-reporter gene. In contrast, an *n*-3 polyunsaturated fatty acid, docosahexaenoic acid, inhibits TLR4 agonist (LPS)-induced up-regulation of the costimulatory molecules, MHC class II, and cytokine production. Similarly, DCs treated with lauric acid show increased T cell activation capacity, whereas docosahexaenoic acid inhibits T cell activation induced by LPS-treated DCs. Together, our results demonstrate that the reciprocal modulation of both innate and adaptive immune responses by saturated fatty acid and *n*-3 polyunsaturated fatty acid is mediated at least in part through TLRs. These results imply that TLRs are involved in sterile inflammation and immune responses induced by nonmicrobial endogenous molecules. These results shed new light in understanding how types of dietary fatty acids differentially modulate immune responses that could alter the risk of many chronic diseases. ***The Journal of Immunology,*** **2005, 174: 5390–5397.**

The TLR plays a critical role in host defense against microbial infection. TLRs function as a sensor of infection by recognizing conserved molecular structures of microbial origin, and induce innate and adaptive immune responses that are required to eliminate infecting pathogens (1–3). So far, 11 TLRs have been identified in humans and mice and are ubiquitously expressed in tissues. In addition to TLR agonists derived from bacterial and viral origins, several nonmicrobial agonists have also been implicated. These agonists include heat shock proteins 60 and 70 (4–6). The activation of TLR4 by heat shock proteins was shown to disappear if contaminated LPS was exhaustively removed (7). Additional agonists include type III repeat extra domain A of fibronectin, Taxol, and saturated fatty acids (8–10). Such a broad spectrum of TLR4 agonists leads to the speculation for the possible presence of physiologic endogenous agonists, and much broader roles of TLRs than we currently understand.

Results from our previous studies demonstrated that saturated fatty acids activate TLR2 dimers and TLR4, whereas unsaturated fatty acids inhibit the agonist-induced activation of TLRs (9, 11, 12). These results suggest that certain TLRs can function as a sensor for the delicate balance between saturated and unsaturated fatty acids. These results further suggest that the induction of Toll-mediated immune responses can also be affected by the balance between saturated and unsaturated fatty acids, which in turn can be altered by the types of dietary fat consumed.

Dendritic cells (DCs)[3] are the professional APCs that uptake Ag in peripheral tissues and migrate to the draining lymph nodes where they stimulate naive T lymphocytes. DCs undergo the maturation process for efficient activation of T cells. Activation of TLRs provides critical signals for the maturation of DCs that is accompanied by cytokine production, up-regulation of costimulatory molecules, and enhanced ability to activate naive T cells (13, 14). Thus, the activation of TLRs in DCs is critical in bridging the innate and adaptive immune responses. It has been well documented that types of dietary fatty acids can differentially modulate immune responses (15–17). However, the mechanisms for such modulation are not well understood. Therefore, we investigated whether saturated and polyunsaturated fatty acids alter immune responses as a functional consequence of the modulation of TLR signaling pathways and target gene expression in DCs.

Materials and Methods

Mice

Female BALB/c, DO11.10 on a BALB/c background, C3H/HeJ, or C3H/HeOUJ mice 6–12 wk of age were purchased from The Jackson Laboratory. Animals were housed in the animal facility in bioclean racks in accordance with the guidelines from the Association for Assessment and

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Received for publication October 13, 2004. Accepted for publication February 24, 2005.

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[1] This work was supported by Grants DK41868 and CA75613 from the National Institutes of Heath, Grant 97-37200-4258 from the U.S. Department of Agriculture, Grant 98A0978 from the American Institute for Cancer Research, and by program funds from the Western Human Nutrition Research Center.

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[3] Abbreviations used in this paper: DC, dendritic cell; DHA, docosahexaenoic acid; TRIF, Toll/IL-1R domain-containing adapter inducing IFN-β; IRF3, IFN regulatory factor 3; ISRE, IFN-stimulated regulatory element; DN, dominant negative; Hsp, heat shock protein.

0022-1767/05/$02.00

Accreditation of Laboratory Animal Care International and with Institutional Animal Care and Use Committee approval.

Reagents and Abs

Lauric acid sodium salt was purchased from Nu-Chek Prep. Purified LPS was purchased from List Biological Laboratories. Docosahexaenoic acid (DHA) sodium salt was purchased from Sigma-Aldrich. CD16/CD32 blocking Ab was purchased from BD Pharmingen. Fluorochrome-conjugated Abs to CD11c, MHC class II (I-A^d), CD40, CD80, and CD86 were purchased from BD Pharmingen. OVA peptide 323–339 was purchased from Bachem. Mouse inflammation cytometric bead arrays for the analysis of cytokines were purchased from BD Pharmingen.

Plasmids

The genomic upstream regulatory sequence of murine CD86 was cloned using the mouse GenomeWalker kit (BD Clontech) with modifications. We performed two rounds of PCR using two sets of gene-specific primers (GSP) based on the published mouse CD86 cDNA sequence and the working draft (February 2003 assembly) of mouse genomic sequence from the University of California, Santa Cruz genome bioinformatics website ⟨www.genome.ucsc.edu/⟩. The primer sequences were: For1, 5′-agtagatgcagag gcccacccccaaacgtt; GSP1, 5′-caccttcccttctgcgctctca; For2, 5′-tagaagcta gaggagtcaaggataccag; GSP2, 5′-agatctgcgaccacttaccatctggggtccatc. *Bgl*II site (underlined) was added to the GSP2 primer to facilitate the cloning. The PCR amplification was done using BD Advantage 2 polymerase mix (BD Clontech) and *Pvu*II library as the template for the first round and a 1/15 dilution of the first round PCR as a template for the second round of PCR. The PCR conditions consisted of 1 min at 95°C, seven cycles of 2 s at 95°C, 3 min at 70°C followed by 30 cycles of 2 s at 95°C, 3 min at 67°C with final extension of 7 min at 72°C using iCycler (Bio-Rad). A single band of ~1.3 kb was obtained after two rounds of PCR and cloned into pCR2.1 TA cloning vector (Invitrogen Life Technologies). The *Kpn*I-*Bgl*II fragment from the resulting construct was obtained and ligated to precut pGL3-basic reporter vector (Promega) generating the mouse CD86 promoter luciferase reporter construct. The construct was confirmed by DNA sequencing. The nucleotide sequence has been submitted to GenBank nucleotide sequence database (accession no. AY741809).

The luciferase reporter plasmid containing the promoter of human CD86 (−1247/+45) was from Dr. N. Suciu-Foca (Columbia University, New York, NY). Heat shock protein (Hsp)70 β-galactosidase reporter plasmid was from Dr. R. Modlin (University of California, Los Angeles, CA). Mouse pDisplay-TLR4 (P>H) was obtained from Dr. L. Hajjar (University of Washington, Seattle, WA). A dominant negative (DN) mutant of Toll/IL-1R domain-containing adapter inducing IFN-β (TRIF(ΔNΔC)) was obtained from Dr. S. Akira (Osaka University, Osaka, Japan). A DN mutant form of MyD88 (MyD88(ΔDD)) was kindly provided by Dr. J. Tschopp (University of Lausanne, Lausanne, Switzerland). A DN mutant of IFN regulatory factor 3 (IRF3(DBD)) was provided by Dr. G. Cheng (University of California, Los Angeles, CA). A DN mutant of inhibitor NF-κB (pCMV4-IκBα(ΔN)) was provided by Dr. D. Ballard (Vanderbilt University, Nashville, TN). All DNA constructs were prepared in large scale using EndoFree Plasmid Maxi kit (Qiagen) for transfection.

Transient transfection and luciferase assays

Transient transfection and luciferase assays were performed as described in our previous studies (9, 11, 12, 18, 19). Briefly, RAW264.7 cells were cotransfected with a luciferase plasmid containing CD86 promoter and Hsp70 β-galactosidase plasmid as an internal control using SuperFect transfection reagent (Qiagen) according to the manufacturer's instructions. Various expression plasmids or corresponding empty vector plasmids for signaling components were cotransfected. The total amount of transfected plasmids was equalized by supplementing with the corresponding empty vector to eliminate the experimental error from the transfection itself. Luciferase and β-galactosidase enzyme activities were determined using the Luciferase Assay System and β-galactosidase Enzyme System (Promega) according to the manufacturer's instructions. Luciferase activity was normalized by β-galactosidase activity to correct for the transfection efficiency.

Cell culture

RAW264.7 cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% (v/v) heat inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies). Cells were maintained in an incubator at 37°C and 5% CO_2.

Bone marrow cells were isolated from the femur of BALB/c, C3H/HeOUJ, or C3H/HeJ mice. RBC were lysed using a hypotonic salt solution. Remaining bone marrow cells were cultured in RPMI 1640 containing 10% (v/v) heat inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies). GM-CSF (10 ng/ml; Calbiochem) was supplemented to differentiate bone marrow cells to DCs. On day 2, cells were supplemented with additional culture media containing FBS, penicillin, streptomycin, and GM-CSF. On day 5, loosely attached cells were harvested, washed, resuspended in 0.25% FBS RPMI 1640, and used for ensuing experiments.

Expression of MHC class II and costimulatory molecules

Bone marrow-derived DCs, seeded at 1×10^5 cells per well in 96-well plates, were treated with vehicle, lauric acid (75 μM), LPS (10 ng/ml), or LPS with DHA (15 μM). Cells were incubated at 37°C with 5% CO_2. Cell supernatants were collected after 24 and 48 h of culture, and used for cytokine measurement. Leftover cells were stained for costimulatory molecule expression. Cells were stained with fluorochrome-conjugated Abs for 30 min on ice and then washed twice in PBS containing BSA and sodium azide. Cells were analyzed by flow cytometry using a FACSCalibur and CellQuest software (BD Biosciences).

In vitro T cell activation

Bone marrow-derived DCs were plated at 1×10^5 cells per well in 96-well plates and then treated with vehicle, lauric acid, LPS, or LPS with DHA for 48 h. DCs were then washed and irradiated at 1350 rads using a ^{137}Cs irradiator (Institute of Toxicology and Environmental Health, University of California, Davis, CA). T cells were purified from the lymph nodes and spleen of DO11.10 transgenic mice on a BALB/c background. Briefly, RBC were lysed, and then $CD4^+$ T cells were purified using mouse CD4 Dynabeads (Dynal Biotech). Attached beads were removed using the CD4 DetachaBead (Dynal Biotech). Purified T cells were then added to the irradiated DC culture along with the OVA peptide 323–339 (Bachem) and incubated for 48 h. For the last 24 h of culture, 1 μCi [^{3}H]thymidine (ICN Pharmaceuticals) was added per well. Thymidine incorporation was determined by liquid scintillation counting.

Measurement of cytokine production

Cytokine levels in DC culture supernatants were measured using the Mouse Inflammation Cytometric Bead Array (BD Pharmingen) according to the manual provided.

Statistical analysis

Data were presented as the mean and SE of means for all treatment groups. The data were subjected to one-way Student's *t* test or one-way ANOVA followed by Dunnett's test or a single degree of freedom contrast to determine whether the means differed significantly from each other or the vehicle. In all cases, a value of $p < 0.05$ was used to specify significance.

Results

Saturated fatty acids and the polyunsaturated fatty acid, DHA, reciprocally modulate the expression of MHC class II and costimulatory molecules in bone marrow-derived DCs

Results from our previous studies demonstrated that saturated fatty acids activate TLR4, whereas unsaturated fatty acids inhibit the activation in the macrophage cell line, RAW264.7 (9, 11, 19). The activation of TLRs in DCs induces maturation characterized by the up-regulation of MHC class II, costimulatory molecules, cytokine production, and increased ability to activate naive T cells (1, 2). Therefore, we determined whether fatty acids differentially affect the maturation of DCs as a functional consequence of the modulation of TLRs. We investigated whether saturated fatty acids up-regulate the expression of the costimulatory molecules and MHC class II, and whether the polyunsaturated fatty acid, DHA, inhibits the saturated fatty acid (lauric acid)-induced up-regulation of CD86 in DCs. CD11c is a surface marker for differentiated DCs. Therefore, the level of costimulatory molecule expression was determined out of $CD11c^+$ stained cells. The results showed that most saturated fatty acids (capric, lauric, and palmitic) up-regulated CD86 expression (Fig. 1*A*). Lauric acid was the most potent saturated fatty acid among the fatty acids tested in up-regulating CD86 expression. Therefore, we used lauric acid as a representative saturated fatty acid in this study.

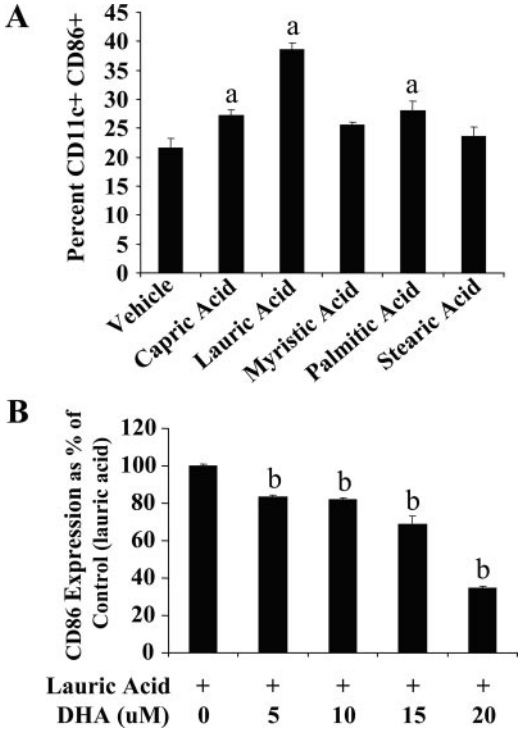

FIGURE 1. Saturated fatty acids up-regulate the expression of the costimulatory molecule CD86, but DHA inhibits the saturated fatty acid (lauric acid)-induced up-regulation of CD86. Primary bone marrow cells were isolated from BALB/c mice and differentiated with 10 ng/ml GM-CSF for 5 days. *A*, Differentiated cells were then treated for 48 h with vehicle or 75 μM of saturated fatty acids or differentiated cells were treated with 75 μM of lauric acid in the presence of different concentrations of DHA (*B*). Cells were washed and stained for CD11c and CD86. Values are mean ± SEM ($n = 3$). Data are representative of three replicate experiments. a, Significantly different from the control media by $p < 0.05$ using one-way ANOVA followed by Dunnett's test; b, significantly different from the lauric acid alone control by $p < 0.05$ using one-way ANOVA followed by Dunnett's test.

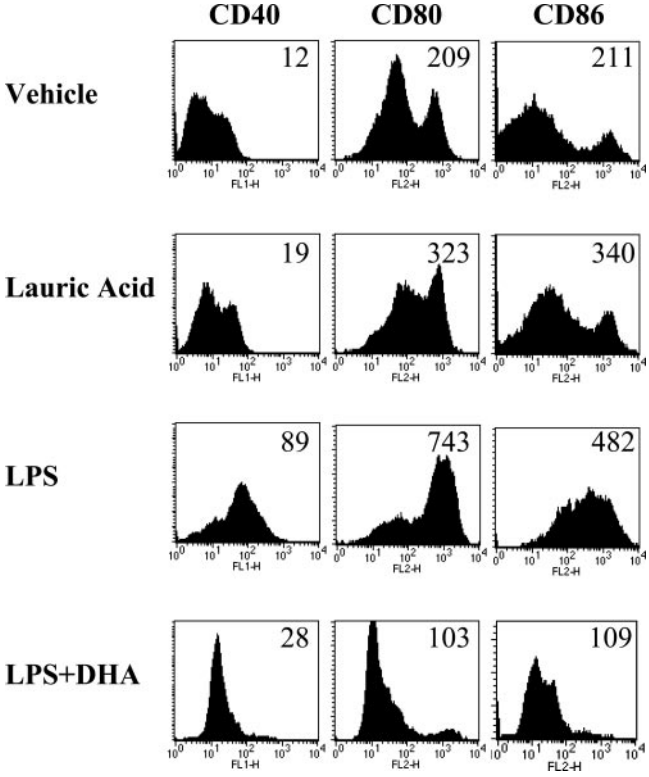

FIGURE 2. Reciprocal modulation of costimulatory molecule expression by lauric acid and DHA in bone marrow-derived DCs. Primary bone marrow cells were isolated from BALB/c mice and differentiated with 10 ng/ml GM-CSF for 5 days. Differentiated cells were then treated for 48 h with vehicle, 75 μM lauric acid, 10 ng/ml LPS, or LPS with 15 μM DHA. Cells were washed and stained for CD11c, CD40, CD80, and CD86. Shown are histograms of costimulatory molecules gated out of the $CD11c^+$ population. Mean fluorescence intensity (*inset upper right*) is indicated for each sample. Data are representative of more than three replicate experiments.

Results from our previous studies showed that DHA was the most potent inhibitor for the agonist-induced activation of TLR4 or TLR2 among unsaturated fatty acids tested (19). Thus, DHA was used as a representative polyunsaturated fatty acid in this study. DHA inhibited lauric acid-induced up-regulation of CD86 expression in a dose-dependent manner (Fig. 1*B*).

Lauric acid also up-regulated the surface expression of other costimulatory molecules such as CD40 and CD80 in bone marrow-derived primary DCs (Fig. 2). Similarly, LPS, a bacterial TLR4 agonist, up-regulated all costimulatory molecules analyzed (Fig. 2). In contrast, DHA inhibited the LPS-induced up-regulation of CD40, CD80, and CD86 expression (Fig. 2). These reciprocal effects of lauric acid and DHA on the expression of costimulatory molecules are consistent with our previous results demonstrating the opposite modulation of TLR4 activation by lauric acid and DHA in murine macrophages (9, 11, 19).

In addition to the expression level of costimulatory molecules, we also determined the percentage of the DC population that expresses MHC class II and costimulatory molecules. Treatment with lauric acid or LPS increased the percentage of $CD11c^+$ MHC class II^+ cells (49.20 and 64.86%, respectively, vs 27.36% with vehicle). Conversely, treatment with DHA decreased the $CD11c^+$ MHC class II^+ population induced by LPS treatment (54.14 vs 64.86% with LPS alone) (Table I). Lauric acid increased the percentage of the DC population double positive with CD11c and the costimulatory molecule CD40, CD80, or CD86. The population of $CD11c^+CD40^+$, $CD11c^+CD80^+$, and $CD11c^+CD86^+$ was increased in the lauric acid group compared with the vehicle group (13.7 vs 10.2%, 58.5 vs 39.4%, and 32.4 vs 24.7%, respectively) (Table I). Treatment with LPS also resulted in increased double positive populations of DCs (Table I). In contrast, the population of $CD11c^+CD40^+$, $CD11c^+CD80^+$, or $CD11c^+CD86^+$ cells increased by LPS was reduced by DHA in DCs (60.6 vs 35.7%, 80.5 vs 14.2%, and 58.3 vs 46.3%, respectively) (Table I). Collectively, these results demonstrate that the saturated fatty acid, lauric acid, up-regulates the expression of the MHC class II and costimulatory molecules whereas the polyunsaturated fatty acid, DHA, inhibits LPS-induced up-regulation of MHC class II and costimulatory molecules in DCs.

Transcriptional activity of CD86 promoter is differentially modulated by lauric acid and DHA

Next, to investigate whether the modulation of costimulatory molecule expression by fatty acids occurs at the transcriptional level, and whether this modulation is mediated through TLR4 activation, we determined the transcriptional activity of the CD86 promoter regulated by lauric acid and DHA using the luciferase reporter gene assay. A comparison of the murine and human CD86 promoter regions showed significant similarities in putative transcription factor binding sites. Both promoters have NF-κB and IFN-stimulated regulatory element (ISRE) sites, which were previously

Table I. *Lauric acid and LPS up-regulate MHC class II and costimulatory molecule expression in primary bone marrow-derived DCs, but DHA inhibits LPS-induced MHC class II and costimulatory molecule expression*[a]

Treatment	Percentage of Cell Population ± SEM			
	MHC Class II$^+$	CD40$^+$	CD80$^+$	CD86$^+$
Vehicle	27.36 ± 0.39	10.22 ± 0.54	39.44 ± 1.02	24.71 ± 0.52
Lauric acid	49.20 ± 0.85*	13.78 ± 0.39	58.52 ± 1.25*	32.43 ± 0.33*
LPS	64.86 ± 0.92*	60.67 ± 1.87*	80.56 ± 0.57*	58.34 ± 0.66*
LPS+DHA	54.14 ± 0.72**	35.76 ± 6.41**	14.29 ± 2.02**	46.31 ± 2.61**

[a] Primary bone marrow cells were isolated and differentiated for 5 days in the presence of 10 ng/ml GM-CSF. Following differentiation, cells (1×10^5) were cultured in RPMI 1640 media with vehicle, 75 μM lauric acid, 10 ng/ml LPS, or LPS with 15 μM DHA. Cells were treated for 48 h and then stained for CD11c, MHC class II, CD40, CD80, and CD86 expression. The percentage of the cell population double positive for CD11c and MHC class II, CD40, CD80, or CD86 was determined by flow cytometry. Values are mean ± SEM ($n = 3$). These data are representative of more than three separate experiments. Significant difference from vehicle control (*, $p < 0.05$ using one-way ANOVA followed by a single degree of freedom contrast). Significant difference from LPS alone treatment (**, $p < 0.05$ using one-way ANOVA followed by a single degree of freedom contrast).

shown to be activated by saturated fatty acids (Fig. 3*A*) (12). The murine macrophage cell line RAW264.7 was transfected with the murine or human CD86 promoter-luciferase reporter plasmid and then stimulated with vehicle, lauric acid, LPS, or LPS with DHA. Lauric acid as well as LPS increased the transcriptional activity of both murine and human CD86 promoters (Fig. 3*B*). However, DHA inhibited LPS-induced expression of both murine and human CD86-luciferase reporter genes (Fig. 3*B*). Next, to determine whether lauric acid-induced CD86 expression is mediated through TLRs, RAW264.7 cells were transfected with a DN mutant of TLR4, or downstream signaling components. Lauric acid-induced murine and human CD86-luciferase expression was significantly decreased when TLR4 DN mutant was cotransfected (Fig. 3*C*). This result suggests that lauric acid-induced CD86 expression is at least partly mediated through TLR4.

TLR4 activates two different downstream signaling pathways: MyD88-dependent and MyD88-independent (TRIF-dependent) pathway. The MyD88-dependent pathway culminates in the activation of NF-κB and MAPK, and the expression of cytokines. The activation of TRIF pathway leads to the activation of IRF3, delayed activation of NF-κB, and in turn, the expression of type I IFNs including IFN-β. Therefore, we next determined the TLR4 downstream signaling pathways mediating lauric acid-induced CD86 expression. The DN mutant of TRIF suppressed both murine and human CD86 promoter activity induced by lauric acid, whereas MyD88 (DN) did not inhibit the human CD86 promoter activity and only slightly inhibited the murine CD86 promoter (Fig. 3*C*). However, cotransfection of MyD88 (DN) and TRIF (DN) resulted in more pronounced inhibition of the promoter activity as compared with individual DN. These results suggest that lauric acid-induced CD86 expression in macrophages is primarily dependent on the TRIF-mediated pathway. Lauric acid-induced CD86 expression was inhibited by the DN mutant of IκBα (Fig. 3*C*). This implies that the activation of NF-κB is at least partially responsible for the saturated fatty acid-induced CD86 up-regulation. The activation of IRF3 through TRIF pathways is known to induce the expression of target genes with the ISRE binding site in the promoter regions (20, 21). Both murine and human CD86-luciferase activity induced by lauric acid was inhibited by the IRF3 (DN) (Fig. 3*C*). Putative NF-κB and ISRE sites are present in both murine and human CD86 promoter regions (Fig. 3*A*). Together, these results suggest that the modulation of lauric acid-induced expression of CD86 is at least in part mediated through TLR4 activation and TRIF signaling pathways that activate both NF-κB and IRF3.

CD11c expression is down-regulated by lauric acid and LPS, whereas DHA reverses the LPS-induced down-regulation of CD11c expression in DCs

CD11c is a cell surface marker that is expressed on differentiated, but immature, DCs. It functions as a complement receptor that can facilitate phagocytosis of pathogens. Following DC maturation, CD11c is down-regulated. In contrast, as DCs undergo the maturation processes, the surface expression of costimulatory molecules is increased. This down-regulation of CD11c is another hallmark of DC maturation (22). Therefore, we determined whether the surface expression of CD11c is differentially modulated by lauric acid and DHA in primary DCs that were differentiated from bone marrow cells for 5 days with GM-CSF. Treating the differentiated DCs with lauric acid led to decreased expression of CD11c; 23.3% of cells were CD11c positive compared with 49.4% of the vehicle control (Fig. 4). LPS, a TLR4 agonist with microbial origin, also decreased CD11c expression; 10.2% compared with the 49.4% vehicle control (Fig. 4). However, DHA reversed LPS-induced down-regulation of CD11c. In the cells treated with LPS and DHA together, 37.3% were CD11c$^+$ compared with the LPS alone group, which had 10.2% CD11c$^+$ (Fig. 4). These results further demonstrate that the saturated fatty acid, lauric acid, induces DC maturation whereas the polyunsaturated fatty acid, DHA inhibits LPS-induced DC maturation.

Lauric acid increases, but DHA suppresses LPS-induced, inflammatory cytokine production during DC maturation

Agonist-induced activation of TLRs leads to the activation of the downstream signaling molecules including NF-κB, MAPKs, and IRF3, and the expression of cytokines. Thus, we determined whether the expression of cytokines is similarly modulated by the fatty acids during the maturation of DCs. IL-12p70 is responsible for driving CD4$^+$ T cells to a Th1 response, and its production by DCs correlates with the functional maturation of DCs (23). Unlike IL-12p70, IL-6 does not promote DC maturation, but induces acute phase responses (23). Bone marrow-derived DCs were cultured in the presence of vehicle, lauric acid, LPS, or LPS with DHA. Cell supernatants were collected after 24 h of treatment, and analyzed for IL-12p70 and IL-6 using a cytometric bead array. The results showed that both lauric acid and LPS stimulated the production of IL-12p70 and IL-6 in DCs (Fig. 5, *A* and *B*). However, DHA inhibited LPS-induced IL-12p70 and IL-6 production in DCs (Fig. 5*B*). These results demonstrate that lauric acid induces the production of cytokines that favor Th1 immunity and promote DC maturation, whereas DHA inhibits LPS-induced cytokine production in DCs.

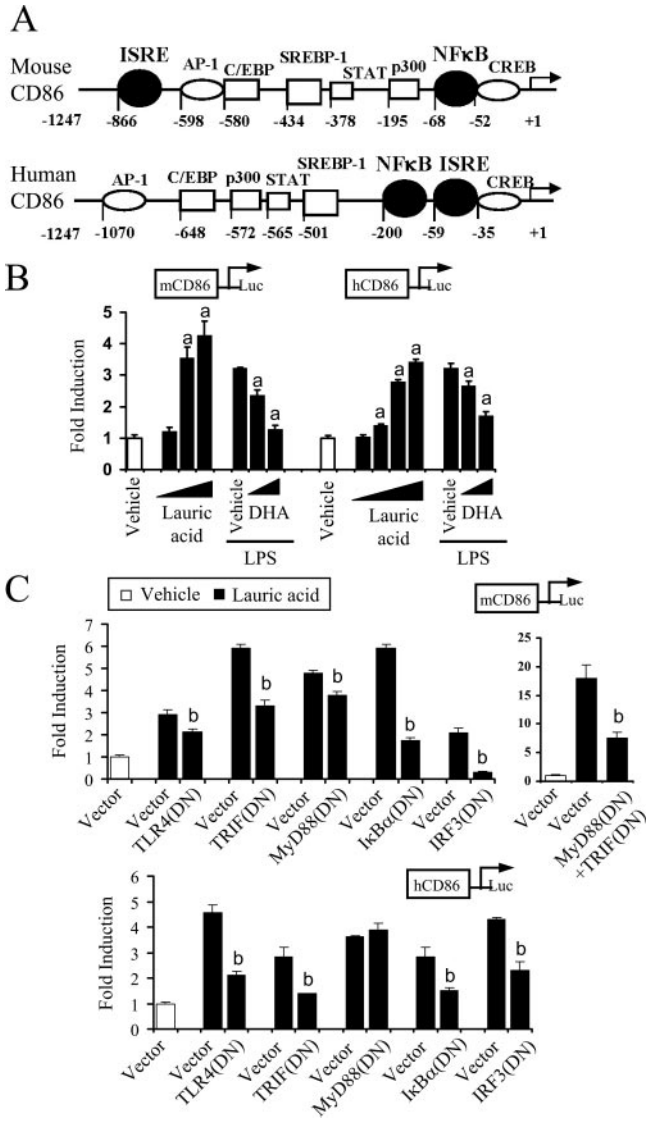

FIGURE 3. Lauric acid induces, but DHA inhibits LPS-induced CD86 promoter activation through TLR signaling. *A*, Schematic illustration of putative transcription factor binding sites in both human and mouse CD86 promoter regions is shown. The nucleotide sequences upstream from the transcription start sites of the mouse and human CD86 genes (accession nos. BC013807 and U04343, respectively) were searched for transcription factor binding sites using the MATCH program and the TRANSFAC database version 6.0. The position of the best matrix match for each transcription factor binding site is shown. *B*, RAW264.7 cells were transfected with human or murine CD86 promoter-luciferase reporter plasmid. After 24 h, cells were stimulated with different doses of lauric acid (5, 20, and 75 μM for mouse CD86; 10, 20, 50, and 75 μM for human CD86), LPS 50 ng/ml, or LPS with DHA (5 or 10 μM) for 18 h. *C*, RAW264.7 cells were cotransfected with CD86 reporter gene and various expression plasmids as indicated. As a vector control, pDisplay for TLR4 (DN) (TLR4 (P>H)), pCMV for TRIF (DN) (TRIF(ΔNΔC)) and IκBα (DN) (IκBα(ΔN)), pcDNA for MyD88 (DN), and pEBB for IRF3(DN) (IRF3(DBD)) were used. After 24 h, cells were stimulated with lauric acid (50 μM), or LPS (25 ng/ml) for 18 h. Values are mean $\pm$ SEM ($n = 3$). a, Significantly different from the vehicle control by $p < 0.05$ using one-way Student's t test.; b, significantly different from corresponding vector + lauric acid by $p < 0.05$ using one-way Student's t test.

Ag-specific T cell activation by DCs is reciprocally modulated by lauric acid and DHA

The activation of TLRs in DCs also enhances the ability to activate naive T cells. Therefore, we determined whether the fatty acids differentially modulate Ag-specific T cell activation as a result of the modulation of MHC class II, costimulatory molecules and cytokine production in DCs. Bone marrow-derived DCs were treated for 48 h with either vehicle, lauric acid, LPS, or LPS with DHA. Treated DCs were then irradiated, and primary naive T cells isolated from DO11.10 mice and Ag, OVA, were added to the culture. T cell activation was determined by [^{3}H]thymidine incorporation. The results showed that lauric acid induced a dose-dependent increase in primary T cell activation by DCs (Fig. 6*A*). DCs treated with LPS also caused an increase in T cell activation compared with DCs treated with vehicle alone. DHA inhibited LPS-induced T cell activation in a dose-dependent manner (Fig. 6*B*). Together, our results demonstrate that the saturated fatty acid, lauric acid, stimulates whereas the polyunsaturated fatty acid, DHA, inhibits LPS-induced T cell activation in the coculture system. This reciprocal modulation of T cell activation by the fatty acids was at least in part mediated through their corresponding modulation of DC maturation that is in turn dependent on the activation of TLRs.

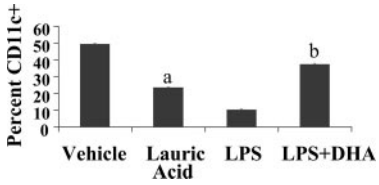

FIGURE 4. CD11c expression is down-regulated by lauric acid and LPS, but up-regulated by DHA in bone marrow-derived DCs. Primary bone marrow cells were differentiated for 5 days in the presence of 10 ng/ml GM-CSF. Differentiated cells were then treated for 48 h with vehicle, 75 μM lauric acid, 10 ng/ml LPS, or 10 ng/ml LPS with 20 μM DHA. Cells were then washed and stained with CD11c and analyzed by flow cytometry. Values are mean $\pm$ SEM ($n = 3$). These data are representative of four separate experiments. a, Significantly different from vehicle control by $p < 0.05$ using one-way ANOVA followed by a single degree of freedom contrast; b, significantly different from LPS alone treatment with a value $p < 0.05$ using one-way ANOVA followed by a single degree of freedom contrast.

Discussion

The results from our previous studies demonstrated that saturated fatty acids activate TLR4 and TLR2 dimers with TLR6 or TLR1, whereas unsaturated fatty acids inhibit LPS-induced or a synthetic lipopeptide palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)propyl)-Ala-Gly-OH (PamCAG)-induced activation of TLR4 or TLR2 dimers, respectively (11, 12). Therefore, we determined whether saturated and polyunsaturated fatty acids reciprocally modulate DC maturation as a functional consequence of the modulation of TLRs. Our results demonstrating up-regulation of costimulatory molecules and MHC class II by lauric acid but down-regulation by DHA in DCs are consistent with our previous results showing the opposite modulation of TLRs and the target gene product inducible cyclooxygenase by lauric acid and DHA in macrophages (11, 12, 19). LPS-induced maturation of bone marrow-derived DCs from TLR4 mutant mice (C3H/HeJ) was severely impaired as compared with that of DCs from wild-type mice (data not shown) indicating that TLR4 plays a critical role in LPS-induced DC maturation. Furthermore, the results from CD86 promoter-reporter gene assays demonstrated the reciprocal modulation of CD86 expression by lauric acid and DHA in a macrophage cell line. Lauric acid up-regulated both human and murine CD86 promoter activity, and this up-regulation was inhibited by the transfection of the DN mutant of TLR4. These results demonstrate that the modulation of CD86 promoter activity by lauric acid is at least in part mediated through

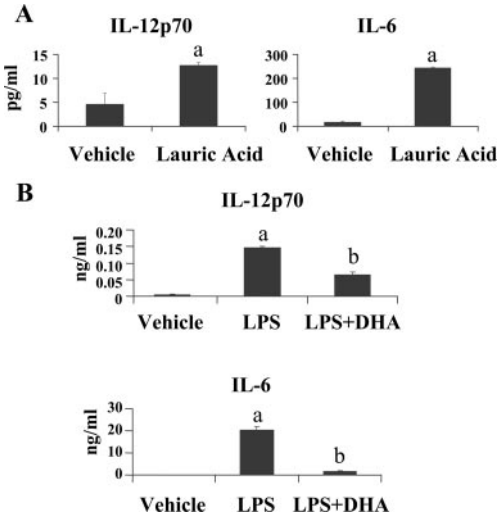

FIGURE 5. The production of IL-12p70 and IL-6 is enhanced by lauric acid but DHA inhibits the LPS-induced cytokine production in bone marrow-derived DCs. Primary bone marrow cells were isolated and differentiated for 5 days in 10 ng/ml GM-CSF. Differentiated cells were then treated for 24 h with vehicle (*A*) or 75 μM lauric acid or 10 ng/ml LPS or LPS with 15 μM DHA (*B*). At 24 h cell supernatants were collected and cytokine production was analyzed using a Mouse Inflammation Cyotmetric Bead Array. Values are mean ± SEM ($n = 3$). These data are representative of three separate experiments. a, Significantly different from vehicle control by $p < 0.05$ using one-way Student's *t* test; b, significantly different from LPS alone treatment by $p < 0.05$ using one-way Student's *t* test.

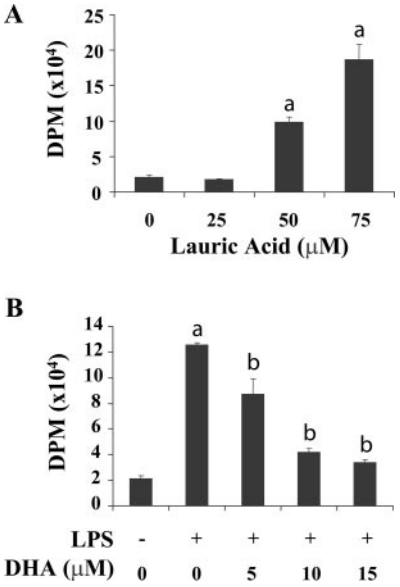

FIGURE 6. Ag-specific T cell activation by DCs is enhanced in a dose-dependent manner by lauric acid, but DHA inhibits the activation induced by DCs treated with LPS. Primary bone marrow cells were isolated and differentiated for 5 days in 10 ng/ml GM-CSF. *A*, Differentiated cells were then treated for 48 h with vehicle, 25, 50, or 75 μM lauric acid. *B*, Differentiated cells were then treated for 48 h with vehicle or 10 ng/ml LPS and 0, 5, 10, or 15 μM DHA. Cells were then washed, irradiated, OVA peptide (323–339) and primary naive T cells derived from DO11.10 mice were added, and cultures were then incubated for 48 h. [^{3}H]Thymidine was added during the last 24 h of the incubation. Values are mean ± SEM ($n = 3$). These data are representative of three separate experiments. a, Significantly different from vehicle control by $p < 0.05$ using one-way ANOVA followed by Dunnett's test; b, significantly different from LPS alone treatment by $p < 0.05$ using one-way ANOVA followed by Dunnett's test.

TLR4. The promoter-reporter gene assay for CD86 was performed in a macrophage cell line (RAW264.7) instead of primary DCs that are unsuitable for the reporter gene assay. Macrophages are another APC and express endogenous CD86 (data not shown). Thus, we reasoned that the promoter-reporter gene assay for CD86 in RAW264.7 cells may qualitatively reflect the promoter activity of the CD86 gene in DCs.

It has been well recognized that the activation of TLRs by molecules with nonmicrobial origins requires rigid validation that the activation is not mediated by contaminating microbial molecules such as LPS. As an example, a controversy arose from the results suggesting that the activation of TLR4 by Hsp60 might be due to LPS contamination (5–7). To address this issue, we determined whether lauric acid-induced NF-κB activation was abolished by treating the cells with polymyxin B. The results showed that polymyxin B up to 50 μg/ml (400 U/ml) did not inhibit lauric acid-induced NF-κB activation, whereas polymyxin B (5 μg/ml, 40 U/ml) maximally inhibited LPS-induced NF-κB activation (data not shown). Recently, Valentinis et al. (24) showed that polymixin B alone at 70 U/ml induced the maturation of human DCs mediated through the activation of Erk1/2. However, our results showed that up to 50 μg/ml polymixin B did not affect NF-κB activation (data not shown). Furthermore, unlike recombinant Hsp60, which is prepared using a bacterial expression system, the purification of fatty acids (Nu-Check Prep) involves alkaline saponification of triglycerides (plant or marine oil) and vacuum distillation. During the saponification, any LPS or lipopeptide would be inactivated because saturated fatty acids acylated in lipid A or lipopeptides will be hydrolyzed. It is well documented that if fatty acids acylated in LPS or lipopeptides are removed, their endotoxic activity is abolished (25–27). Any LPS or lipopeptide would also be eliminated during the vacuum distillation of fatty acids.

The two major downstream signaling pathways activated by TLR4 agonists are mediated by MyD88 and TRIF adaptor molecules. The activation of MyD88 signaling pathways leads to the stimulation of MAPK and NF-κB, and the expression of cytokines and other inflammatory marker gene products including cyclooxygenase-2 and inducible NO synthase. The activation of the TRIF signaling pathways leads to the activation of IRF3 and the delayed activation of NF-κB resulting in the expression of type I IFNs including IFN-β (28–30). The promoter analysis for both human and murine CD86 demonstrates high homology in *cis*-acting sites for putative transcription factors although there is variation in locations of putative transcription factor binding sites. Of particular note is that both the human and murine CD86 promoters contain NF-κB binding sites and IRF3 binding sites (ISRE). The up-regulation of the promoter activity of both human and murine CD86 by lauric acid was inhibited by the DN mutant of IκBα or IRF3 (Fig. 3). DC maturation induced by LPS and poly(I:C) still occurs in MyD88-deficient cells (22). In addition, it was suggested that the expression of CD86 through TLR3 and TLR4 activation is primarily mediated through the TRIF pathway in murine macrophages (31). Our results also showed that the DN mutant of TRIF significantly inhibited lauric acid-induced CD86 promoter activity in murine macrophage cell line (RAW264.7). Together, our results suggest that lauric acid-induced up-regulation of CD86 is at least in part mediated through TRIF pathways derived from TLR4. However, a synthetic lipopeptide (PamCAG), TLR2 agonist, which can activate MyD88 pathways, but not TRIF, can still up-regulate costimulatory molecules in murine bone marrow-derived DCs (data not shown). These results are consistent with other reports using DCs isolated from human PBMC stimulated with

TLR2 agonists (32, 33). These results further suggest that the activation of MyD88-dependent signaling pathway is sufficient to induce the up-regulation of costimulatory molecules, and that the maximum expression of costimulatory molecules may require signals from both MyD88-dependent and -independent pathways. The promoter regions of both human and murine CD86 contain a putative ISRE site (Fig. 3*A*) that can be activated by TRIF-dependent signals, and a NF-κB site that can be activated both by MyD88-dependent and -independent signals.

The production of cytokines (IL-12p70 and IL-6) that are known to be TLR target gene products was also increased by lauric acid suggesting that it is also mediated through TLRs. Because lauric acid can activate TLR2 dimers in addition to TLR4, our results cannot preclude the possibility that lauric acid-induced up-regulation of MHC class II, costimulatory molecules, and cytokines may also be mediated through TLRs other than TLR4 in DCs.

In addition to MHC class II and costimulatory molecule-mediated T cell activation, DCs have instructive roles for eliciting types of adaptive immune responses by secreting a different array of cytokines. It was known that TLRs control the activation of Ag-specific Th type 1 (Th1) and Th2 immune responses. The types of adaptive immune responses stimulated are also dependent on Ag doses and types of TLR ligands (34). *Escherichia coli* LPS (TLR4 agonist) stimulates DCs to secrete cytokines, such as IL-12p70, that drive $CD4^+$ T cells toward a Th1 immune response (32, 35, 36). The ability of DCs to produce IL-12p70 correlates with its functional maturation (23). Our results show that DCs treated with lauric acid secrete more IL-12p70 than DCs treated with vehicle alone. Conversely, DHA inhibits LPS-induced IL-12p70 secretion in DCs. These results suggest that lauric acid promotes DC maturation through increased expression of not only MHC class II and costimulatory molecules but also IL-12p70, whereas DHA exerts opposite effects on these processes. In addition, the results suggest that saturated fatty acids may induce a Th1 immune response similar to LPS in primary DCs, whereas *n*-3 polyunsaturated fatty acids suppress this response. Interestingly, another cytokine reciprocally regulated by the saturated and polyunsaturated fatty acids was IL-6. IL-6 is an important cytokine responsible for the acute phase inflammatory response. IL-6 is known to induce the production of C-reactive protein (37, 38). C-reactive protein has been identified as an important predictor of coronary heart disease (39). This implies the possibility that saturated fatty acids may stimulate the production of C-reactive protein that correlates with increased risk of coronary heart disease.

Finally, the Ag-specific T cell activation by DCs was also reciprocally modulated by lauric acid and DHA reflecting the opposite effects of lauric acid and DHA on costimulatory molecule expression. These results suggest that types of fatty acids can modulate both innate and adaptive immune responses through their effects on the expression of MHC class II, costimulatory molecules, and cytokines. Results from recent studies by other investigators demonstrated that TLR ligands can enhance DC Ag capture and promote activated $CD4^+$ T cell survival by up-regulation of Bcl-x_L (40, 41). These results provide a conceptual possibility that both innate and adaptive immune responses can be modulated by saturated and polyunsaturated fatty acids at multiple steps of immune responses. Whether the Ag capture by DCs and activated $CD4^+$ T cell survival also can be reciprocally modulated by saturated and polyunsaturated fatty acids needs to be determined in future studies.

Polyunsaturated fatty acids can be enzymatically metabolized via lipoxygenase, cyclooxygenase, or cytochrome P450. Recently, it was shown that novel hydroxy series of DHA metabolites possess potent anti-inflammatory effects (42). Whether the inhibitory effects of DHA on agonist-induced TLR activation and consequent cellular responses are exerted by DHA itself or its enzymatic metabolites needs to be determined in future studies.

Together, our results demonstrate that the saturated fatty acid, lauric acid, stimulates DC maturation and consequent Ag-specific T cell activation, but the *n*-3 polyunsaturated fatty acid, DHA, inhibits the LPS-induced DC maturation and T cell activation. This modulation is mediated at least in part through TLRs. Our results provide new insight on the mechanism for the modulatory roles of different dietary fatty acids on both innate and adaptive immune responses. Currently, TLRs are recognized as evolutionarily conserved receptors involved in detection and host defense against invading microbial pathogens. Our results presented in this study and from previous studies suggest that endogenous molecules of nonmicrobial origin, which include fatty acids, can modulate the activation of TLRs. The profound implication of these results is that TLRs may be involved in sterile inflammation and immune responses. Inflammation is now recognized as one of the key underlying etiologic conditions for the development of many chronic diseases. Reciprocal modulation of TLR-mediated immune responses by different fatty acids can shed a new light in understanding how different types of dietary fatty acid modify the risk of many chronic diseases.

Acknowledgments

We thank Lab Animal Resources (University of California, Davis, CA) for their care of the animals. We thank the Institute of Toxicology and Environmental Health (University of California, Davis, CA) for use of the ^{137}Cs irradiator. We also thank Debra Standridge and Kristina Yekta for technical assistance, and Bruce Mackey for assistance with the statistical analysis of the data.

Disclosures

The authors have no financial conflict of interest.

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